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Partitioning and antioxidant action of the water-soluble antioxidant, Trolox, between the aqueous and lipid phases of phosphatidylcholine membranes: ^{14}C tracer and product studies

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Abstract

The water-soluble antioxidant, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic (Trolox), (4-^{14}C)-labelled, was used to trace its location in the aqueous and lipid phases of liposomes. Trolox was found to partition 20 to 25% into the lipid phase of multilamellar (MLV) and 38–46% into the lipid phase of unilamellar (ULV) egg lecithin liposomes. Trolox and its oxidation products partition readily (40%) into the lipid phase of dilinoleoylphosphatidylcholine (DLPC) MLV liposomes during inhibited peroxidation, thermally initiated by azo-bis(2,4-dimethylvaleronitrile) (ADVN). The time-course of the consumption of Trolox during peroxidation of DLPC liposomes, initiated by ADVN, was followed by separation and analyses of [4-^{14}C]Trolox and its oxidation products. Such studies showed that the consumption of Trolox followed the profile of the inhibition of oxygen uptake. This indicates that Trolox can be used in quantitative studies of membrane peroxidation; for example, to measure the rate of chain initiation (R_i). The product distribution of hydroperoxides, the 9- and 13-*cis,trans* (c,t) and *trans,trans* (t,t) isomers, formed during inhibited peroxidation of linoleate, in DLPC and methyl linoleate in dimyristoyl PC (DMPC) liposomes was determined by HPLC of the derived hydroxy methyl esters. The c,t/t,t (kinetic/thermodynamic) ratios were related to the antioxidant activity of the inhibitors. Both Trolox and α-tocopherol (vitamin E) gave relatively high initial c,t/t,t ratios (6.6 and 7.1) during inhibited peroxidation of DLPC, initiated by water-soluble azo-bis(2-amidinopropane · HCl) (ABAP). High initial c,t/t,t ratios (6.2) were also observed for α-tocopherol-inhibited peroxidation of DLPC liposomes, initiated by lipid-soluble ADVN. On the other hand, the combination of Trolox with ADVN-initiated peroxidation of DLPC or of methyl linoleate in DMPC gave relatively low initial c,t/t,t ratios of 3.5 and 1.3. These results are interpreted in terms of the relative hydrogen atom donating ability of the antioxidants and the homogeneity of the system used. The 9/13 ratios of hydroperoxides were constant (0.9 to 1.0) in all experiments and did not give evidence for preferential trapping by Trolox of peroxyls at the 9-position.

Keywords: Water-soluble antioxidant; Trolox; Vitamin E; Lipid hydroperoxide; Antioxidant activity

1. Introduction

Nature provides a number of water-soluble antioxidants which inhibit membrane damage by acting as anti-peroxidizing agents in trapping oxygen-centred radicals through a free radical chain-breaking mechanism. Considerable interest over the past decade has been devoted to such natural antioxidants as vitamin C [1–3], uric acid [4–6], and natural thiols [7–10]. Since its synthesis twenty years ago [11], the synthetic phenolic antioxidant, Trolox, has been examined for its antioxidant action by a number of investigators in a variety of systems. The structure of Trolox (**1**) exhibits the requirements for effective antioxidant action; namely, it is a hindered phenol with a *para* ether oxygen in a ring system so that the structure possesses the requirement for stereoelectronic stabilization [12] of the resulting phenoxyl radical, **2**, on hydrogen atom transfer to an active oxygen radical, such as peroxyl (Scheme 1).

Because of this chromanol structure, which provides the antioxidant activity and the carboxyl group which effects moderate water-solubility, Trolox has advantages over other active antioxidants (e.g., vitamin E) which are only lipid-soluble. For example, Trolox does not have to be incorporated into the lipid membrane by solvent extraction and coevaporation methods; it can be added directly to the intact system. This makes it convenient for studies on

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natural biological systems and for quantitative studies on model systems.

Trolox is known to exhibit antioxidant activity under a variety of conditions in various systems. It is an effective food preservator [13,14]. Trolox inhibits the radical chain oxidation of proteins [15–17] and enzymes [18–23], and prevents the loss of serotonin and dopamine receptor binding sites in animal brain membranes [24,25]. There is evidence that Trolox also provides protection against free radical cell damage of various types, [26–30] especially when 'conjugated' to lactosylphenylpyranoside [31]. The peroxidation of cholesterol in dipalmitoylphosphatidylcholine liposomes is inhibited effectively by Trolox, even when compared to the effect of vitamin E [32]. This suggests that Trolox may be effective against the peroxidation of low density lipoprotein.

Preliminary studies showed that water-soluble initiators and water-soluble antioxidants (e.g., Trolox), as well as lipid soluble ones, can be used for quantitative kinetic measurements of peroxidation of model membranes [33]. Quantitative methods for obtaining antioxidant activities of phenols are based on the termination of peroxyl radicals by Eqs. (1) and (2).

$$\mathrm{ROO}\cdot + \mathrm{ArOH} \overset{k_{\mathrm{inhibition}}}{\rightarrow} \mathrm{ROOH} + \mathrm{ArO}\cdot \qquad (1)$$

$$\mathrm{ROO}\cdot + \mathrm{ArO}\cdot \overset{\mathrm{fast}}{\rightarrow} \text{non} - \text{radical products} \qquad (2)$$

Antioxidant activities are determined by measuring the reduced rate of oxygen uptake by a substrate in the presence of known amounts of inhibitor during the inhibition period until the antioxidant is used up. These methods were used earlier to measure antioxidant activities of a variety of hindered phenols in membranes [34]. However, it was discovered recently that the effect (k_{inh}) of a water-soluble antioxidant, including Trolox, to inhibit peroxidation of phospholipid membranes depends markedly on the charge type of the membrane [35]. Negatively charged membranes, including dilinoleoylphosphatidylcholine (DLPC) at pH 11, were found to undergo uninhibited peroxidation in the presence of Trolox [35] which is reported to be as effective as α-tocopherol at pH 7 [34].

In view of the significance of a water-soluble antioxidant like Trolox in both physical organic and biochemical research on membranes, it is desirable to determine more specifically how and where it operates its antioxidant activity in lipid membranes. Consequently, we now report on the following quantitative results from the behaviour of Trolox in phosphatidyl choline (PC) membranes.

(1) The location of Trolox between the aqueous phase and lipid phase of multi-lamellar vesicles (MLV) and between these phases of unilamellar vesicles (ULV) of egg lecithin is determined by partitioning studies employing ^{14}C-4 radio-labelled Trolox.

(2) The partitioning of [^{14}C]Trolox, together with its products, between aqueous and membrane phases is determined during the peroxidation of dilinoleoylphosphatidylcholine (DLPC) under known and controlled conditions of the rate of free radical chain initiation.

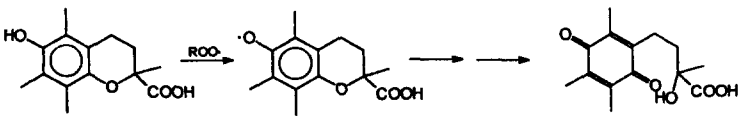
(3) A comparison is made between the conversion of Trolox to its oxidation products and the profile of the induction period (e.g., oxygen uptake) during Trolox-inhibited peroxidation of DLPC liposomes.

(4) Comparisons are made of the linoleate hydroperoxide product distributions during Trolox-inhibited peroxidation of DLPC liposomes with those product distributions during vitamin E-inhibited peroxidation, for peroxidations initiated by a water-soluble initiator azo-bis(2-amidinopropane · HCl) (ABAP) and by a lipid-soluble initiator azo-bis(2,4-dimethylvaleronitrile) (ADVN).

2. Materials and procedures

2.1. Materials

The phospholipids, egg lecithin, DLPC and DMPC were purchased from Avanti Polar Lipids and stored at −30° C. Methyl linoleate was obtained from Nuchek Prep. The azo initiators ADVN and ABAP were purchased from Polysciences. Trolox was purchased from Aldrich Chemicals and radioactive [4-^{14}C]Trolox, specific activity of 57.5 mCi/mg, was a gift from Hoffman-LaRoche. Trolox quinone was prepared by the oxidation of Trolox using a known procedure [13]. α-Tocopherol was purchased from Aldrich Chemicals. Phosphate buffer was prepared from 0.05 M each of NaH_2PO_4 and Na_2HPO_4 in deionized water containing 0.10 mM EDTA. The buffer was passed through a 50–100 mesh Chelex 100 column (Bio-Rad) to remove traces of heavy metal ions.



1 2 3

Scheme 1.

2.2. Liposome preparations

The multilamellar vesicles (MLV) containing known amounts of additives, such as α-tocopherol and ADVN (when required) were prepared by coevaporation from methanol, followed by vortex stirring and by freeze-thaw cycles in liquid nitrogen as reported before [34]. Unilamellar vesicles (ULV) were prepared by several passes of MLV through membranes in a pressure extruder as described earlier [32]. The nature of the MLV and ULV particles was checked by determining the ^{31}P-NMR spectra of samples prepared by these procedures but using Tham buffer at pH 7. The ^{31}P-NMR spectra measured on a JEOL 270 MHz Spectrometer, using pulse sequences as reported [36], exhibited broad anisotropic signals for the MLV samples with chemical shift anisotropies of 45 ppm. The ^{31}P-NMR spectra of extruded samples exhibited single isotropic lines typical of ULV liposomes [32,36].

2.3. Autoxidation / inhibition procedures

The autoxidation kinetic and inhibition studies were carried out at 37° C under 760 torr of oxygen by oxygen uptake methods, using a calibrated dual channel high sensitivity pressure transducer system that was described in detail before [37]. The procedure used varied somewhat with the type of initiation and inhibition. When the water-soluble initiator/inhibitor combination (ABAP/Trolox) was used, a known amount of ABAP in phosphate buffer was added to the DLPC liposomes, equilibrated in the apparatus at 37° C, and when a constant rate of oxygen uptake was observed, a known amount of a solution of Trolox was added to give induction periods long enough (e.g., several hours) to take samples for HPLC analyses or for radiometric counting. When the lipid-soluble initiator/inhibitor combination (α-tocopherol/ADVN) was used, the previously frozen liposomes were equilibrated in the apparatus to 37° C. The error involved in measuring the induction period due to the 'lag time' in such experiments was described earlier [34].

2.4. Partitioning studies using [^{14}C]Trolox

[^{14}C]Trolox was added to a 2.00 ml dispersion of multilamellar egg lecithin (10.7 mM) to make 0.01%, 0.1%, 1% and 4% mol% Trolox samples. The samples were mixed at 250 rpm under nitrogen in a shaker bath set to 37° C. Samples of 200 μl were taken from each dispersion after 45 min and 18 h of continuous shaking and then placed inside an ultrafiltration cone (Cf 50A Amicon Centriflo). The cone was placed inside a centrifuge tube and centrifuged at 2000 × g for 15 min. A 50 μl sample was then removed from the collecting tube and disintegrations per min (DPM) counted, and these are converted to moles from the known amount of [^{14}C]Trolox used. This measurement represents the Trolox dissolved in the water. Another 50 μl sample was taken directly from the cell in the bath and counted. This measurement was the Trolox dissolved in the water and the lipid. The lipid solubilized Trolox was calculated by subtracting the aqueous Trolox from the total Trolox in the sample.

Partitioning of [^{14}C]Trolox during peroxidation of DLPC liposomes was carried out under oxygen in the autoxidation apparatus. A typical procedure used an initial sample of 3.00 ml of multilamellar DLPC ($18.1 \cdot 10^{-3}$ mol) containing $3.80 \cdot 10^{-6}$ mol of initiator (ADVN). In order to follow the oxygen uptake during the inhibition period and take samples throughout for analysis, a preferred procedure involves two identical runs – one is used to measure the uninterrupted oxygen uptake and, the other is used to take samples at convenient times to separate and count the DPM in the aqueous and dispersion phases as above.

2.5. Analyses

In order to follow the consumption of Trolox during inhibition periods, preliminary experiments were performed to determine an optimum ratio of Trolox ($2.60 \cdot 10^{-8}$ mol) to initiator, ADVN ($2.54 \cdot 10^{-6}$ mol), in DLPC ($8.50 \cdot 10^{-3}$ mol) for an induction to last several hours. The experiment was then repeated using [^{14}C]Trolox. Samples (30 μl) were removed after 0, 2, 4 and 7.5 h, and 5 μl of ordinary Trolox (2.80 mM) was added to each to prevent further oxidation during analysis. The samples were applied to the preadsorbant strip of Whatman TLC plates ($LKC_{18}E$) and the chromatogram developed by a mixture of benzene/ethyl acetate/acetic acid (89:9:2). The R_F value for Trolox was 0.71. X-ray film (Kodak-XAR-5) was used to 'photograph' the location of the radioactive components on the TLC plates. The film was exposed to the plates for 24 h, then the TLC was sectioned based on the location of the radiolabels, the adsorbant scraped off and counted for the DPM. The amount of Trolox was calculated from the DPM for each sample. The DPM were counted in liquid scintillation vials on a LKB-Wallac Rackbeta 1211 scintillation counter.

The products from peroxidation of linoleate in DLPC and methyl linoleate in DMPC liposomes were reduced to hydroxy derivatives by Ph_3P, and converted to methyl esters for HPLC analysis by known procedures [36]. The four regio-geometrical isomers were separated on a 4 × 250 mm, 5 μ silica column (Jones Chromatography) using a mixture of hexane/2-propanol/acetone in ratios of 992:4:4 volumes and a flow rate of 1 to 2 ml per min to achieve optimum separation. The isomers elute in the order: (1) 13 *cis, trans*, (2) 13 *trans, trans*, (3) 9 *cis, trans* and (4) 9 *trans, trans*. The peaks areas were corrected for the known molar absorptivities and the ratios calculated as before [36].

Mass spectroscopic analyses were carried out using a Hewlett-Packard 5988A Quadrupole mass spectrometer with the electron energy set at 70 eV. The direct insertion

probe was used to inject Trolox and Trolox quinone. Under these conditions, Trolox exhibited its parent mass (M^+) at $m/z = 250$ (100%) and significant fragment ions at $m/z = 205$ (50% due to loss of HCO_2 and $m/z = 164$ (90%), loss of $CH_2 = CCH_3COOH$, due to a two-bond scission fragmentation. The mass spectrum of the quinone was more complex. The parent mass was observed $m/z = 266$ (19%). Significant fragment ions appeared in the higher mass range at $m/z = 220$ (17%), loss of HCOOH in a cyclic elimination; and at $m/z = 203$ (38%) for loss of water and of HCO_2.

The Trolox quinone was also characterized by its ^{13}C-NMR spectrum. The chemical shifts and assignments are: δ 12.1, 12.3, 12.5, 3-CH_3^s on the ring; δ 26.1, CH_3 at C-2 chain; δ 21.1 and 38.0 CH_2 at C-3 and C-4; δ 74.5 C-2; δ 140.5, 140.9, 141.1, 143.0 for alkene carbons in ring; δ 180 COOH; and δ 187.4, 187.5 for the two C=O groups. Trolox gave the expected ^{13}C-NMR.

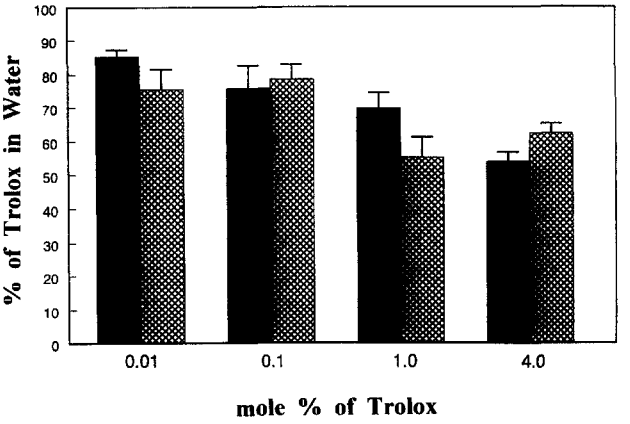


Fig. 2. Trolox (%) in water vs. mol% of Trolox in unilamellar egg lecithin. Respective ratios of Trolox were mixed with $2.13 \cdot 10^{-5}$ mol of egg lecithin in 2.00 ml of 10 mM phosphate-buffered saline. (■) after 45 min of shaking (cross-hatched) after 18 h of shaking. Error bars refer to standard errors of the mean (S.E.).

3. Results

3.1. The partitioning of Trolox between aqueous and lipid phases of egg lecithin liposomes

Results of partitioning studies of Trolox between phosphate buffer (pH 7) aqueous phase and egg lecithin lipid phase when different amounts of Trolox were shaken with multilamellar (MLV) liposomes for known times are outlined in Bar plot profiles produced in Fig. 1. It is clear that the bulk of the Trolox remains in the aqueous phase; ranging from 80%, for 0.01 mol% Trolox introduced, to approx. 75% for 4 mol% Trolox used when the mixture is shaken for an extended time.

Results for partitioning of Trolox under similar conditions as above, but employing unilamellar vesicles (ULV) of egg lecithin, are plotted as before in Fig. 2. The trend of partitioning into ULV is now quite different from that with MLV liposomes. Compared to the MLV egg lecithin, we now find that appreciably more Trolox becomes membrane-bound. With 4 mol% Trolox added initially, up to 38–46% partitions into the lipid phase of ULV liposomes. These results for partitioning of Trolox into MLV and ULV are in general agreement with earlier data using other methods (e.g., spectroscopic) where up to 30% partitioning into egg lecithin ULV was found [35].

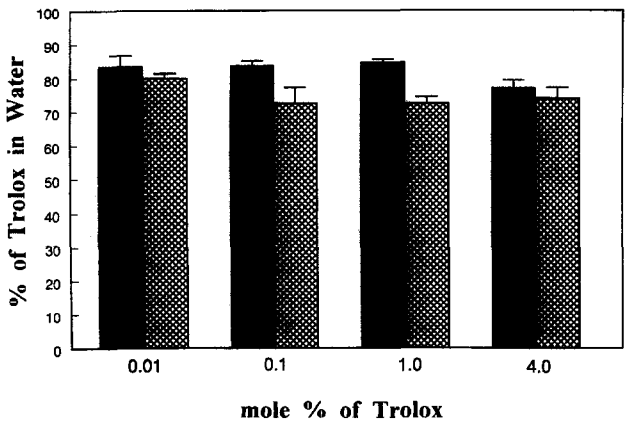


Fig. 1. Trolox (%) in water vs. mol% of Trolox in multilamellar lecithin. Respective ratios of Trolox were mixed with $2.13 \cdot 10^{-5}$ mol of egg lecithin in 2.00 ml of 10 mM phosphate-buffered saline. (■) after 45 min of shaking (cross-hatched) after 18 h of shaking.

3.2. The partitioning of Trolox between aqueous and lipid phases of DLPC liposomes during controlled peroxidation

It was of interest to determine the partitioning of Trolox and its oxidation products during the course of peroxidation of liposomes. For this experiment, the system selected was peroxidation of MLV liposomes of DLPC thermally initiated by the lipid-soluble initiator, azo-bis(2,4-dimethylvaleronitrile) (ADVN). In this system the peroxidation is controlled by a known and controlled rate of initiation (R_i) since the R_i is controlled by the initiator, $R_i = 2k_i e[\text{ADVN}]$, where k_i is the rate constant for initiator decomposition and e is its efficiency, both of which are known [35]. In addition, the free radical peroxidation products from the linoleate chain of DLPC can be determined (vide infra).

The partitioning of Trolox with its oxidation product(s) together with the profile of the resulting inhibition of azo-initiated peroxidation of MLV liposomes of DLPC are illustrated in Fig. 3. The proportion of Trolox and its products which are membrane-bound remains quite constant and at a higher value, approximately 40%, than that found for Trolox in MLV egg lecithin liposomes under argon, approx. 16% (Fig. 1).

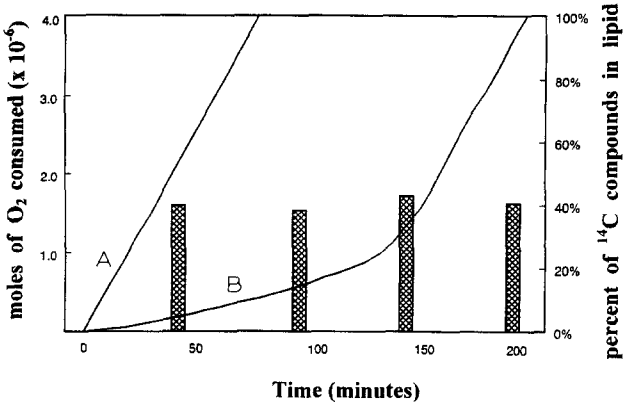


Fig. 3. Trolox partitioning during inhibition run. Four samples were removed at 45, 96, 144 and 198 min after injection of $2.09 \cdot 10^{-8}$ mol of Trolox in 14.2 mg, $1.82 \cdot 10^{-5}$ mol,/ml of DLPC in 3.00 ml of phosphate-buffered saline (10 mM, pH 7.4). ADVN, $1.2 \cdot 10^{-6}$ mol/ml. A, uninhibited reaction; B, inhibited reaction.

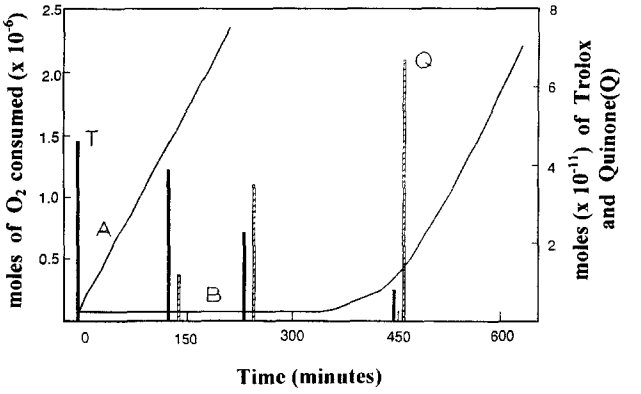


Fig. 4. Decay of Trolox during inhibition. Four samples were removed at 0, 2, 4 and 7.5 h after injection of $2.60 \cdot 10^{-8}$ mol of Trolox (^{14}C) to 2.00 ml of multilamellar dilinoleoylphosphatidylcholine (8.50 mmol) with $2.54 \cdot 10^{-6}$ mol ADVN. The total yield of Trolox was not determined. Mol of Trolox (T) and quinone (Q) shown are amounts determined in each sample taken. A, unhibited reaction; B, inhibited reaction.

3.3. Comparison between the time-course of Trolox oxidation and the induction period during trolox-inhibited peroxidation of DLPC liposomes

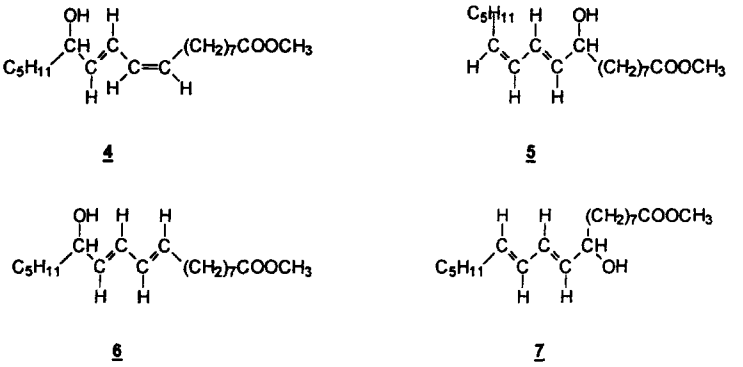
In order for Trolox to be used for quantitative studies of peroxyl radical trapping in aqueous liposomes, the classical Eqs. (1) and (2) for a phenolic inhibitor must apply so that each molecule of Trolox traps two peroxyl radicals. In addition, for quantitative studies in heterogeneous aqueous systems, it is necessary to determine if the Trolox is consumed by the end of the inhibition period of peroxidation in the lipid phase, although it is distributed between the two phases of water and lipid. If both of these conditions are met, the rate of free radical chain initiation (R_i) is given by Eq. (3),

$$R_i = 2[\text{inhibitor}]/\tau \quad (3)$$

where τ is the length of the induction period during which oxygen uptake is suppressed. This relationship is very significant, because it is used to measure the rate of free radical chain initiation (R_i) required for quantitative kinetic studies [33].

We found recently that Trolox does indeed trap two peroxyls radicals per molecule of Trolox during inhibited peroxidation of DLPC liposomes [35]. The present study was carried out to determine the consumption of Trolox during the course of the induction period. This study was carried out using [4-^{14}C]Trolox as in the partitioning studies, then separating by TLC the Trolox ($R_F = 0.71$) from the membrane and Trolox oxidation products and quantitative determination, by the usual counting technique, of the amounts of Trolox and its products during the course of the inhibited peroxidation. A typical result of such a time-course profile is illustrated in Fig. 4. Such results show that Trolox decreases steadily and is essentially all consumed near the end of the induction period (where the lines cross from the slope of the induction period and where the oxygen uptake returns to the original rate), while the amount of the quinone increases.

Our original [^{14}C]Trolox contained an unidentified component which developed more rapidly than Trolox or quinone, **3**, on the TLC sheet ($R_F = 0.83$). The aliquots from oxidation of DLPC containing [^{14}C]Trolox also showed this component initially. This component decayed with time along with Trolox, both to form **3**, and the sum



Scheme 2.

of the counts of this component and Trolox during the run was the same as the quinone formed. The final oxidation product of Trolox in water is known to be the quinone and intermediate oxidation products of Trolox also form the quinone in water [38,39]. The adduct from the Trolox radical, **2**, with peroxyls is expected to hydrolyze rapidly to the quinone, as observed for such adducts with vitamin E [40]. These two sources of the quinone, the adduct with peroxyls and intermediates initially present, would account for the increase yield of **3** compared to the original Trolox (Fig. 4). This does not alter the essential result; namely, that Trolox is consumed by the end of the induction period in our experiments.

3.4. Product studies of linoleate hydroperoxides during inhibited peroxidation(s)

The products of free radical peroxidation of linoleate are the known 9- and 13-hydroperoxy-substituted 9-*cis*,11-*trans*; 10-*trans*,12-*cis*; 9-*trans*,11-*trans*; and 10-*trans*,12-*trans* octadecadienoates, which are characterized by reduction and conversion to the corresponding hydroxymethyl esters (**4**, **5**, **6**, **7** shown in Scheme 2), and the latter are separated and quantified by known HPLC methods [41,42].

These product studies were carried out during inhibited peroxidations of DLPC in MLV liposomes under a variety of conditions. Hydroperoxides were analyzed when peroxidations were initiated by the water-soluble initiator, azo-bis(2-amidinopropane · HCl, ABAP) and inhibited by lipid-soluble α-tocopherol (vitamin E) or by water-soluble Trolox. Similarly, product studies were performed for reactions initiated by the lipid-soluble initiator, azo-bis(2,4-dimethylvaleronitrile) (ADVN) during inhibition by α-tocopherol or by Trolox.

Product analyses for the combination α-tocopherol and ABAP are given as a time-course study of the ratio of *cis / trans* to *trans / trans* (c,t/t,t) products, as well as that of the ratios of substitution at the 9/13 positions as illustrated in Fig. 5. The extrapolated value of the c,t/t,t = 6.6 is attributed to the good hydrogen atom donating ability of vitamin E which traps the c,t or kinetic products before extensive isomerization to the more stable (thermodynamic) t,t isomers. This is in general agreement with earlier observations when higher c,t/t,t ratios were found for much higher vitamin E contents [42].

Results from product analyses using the combination of Trolox with initiation of peroxidation of DLPC by ABAP are illustrated in Fig. 6. The extrapolated c,t/t,t ratio to zero time = 7.1 is similar to that found for vitamin E. However, the slope of the time profile with Trolox is more shallow, −0.16 versus −0.58 with vitamin E, with a sudden drop of the c,t/t,t ratio immediately following the end of the inhibition period. These results indicate that Trolox is a better inhibitor than vitamin E under these conditions. This was found to be the case during Trolox or

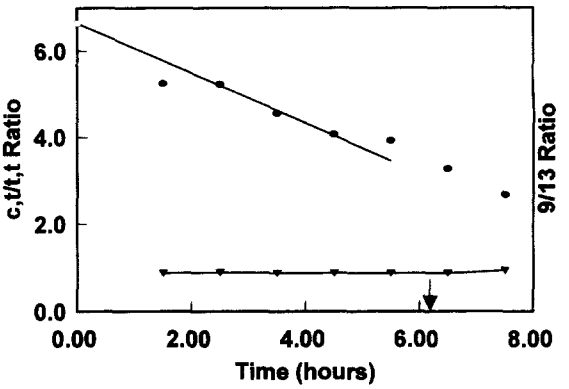


Fig. 5. *Cis,trans* to *trans,trans* (c,t/t,t) linoleate hydroperoxides (●) and 9/13 ratios (▼), from peroxidation of DLPC, initiated by ABAP and inhibited by vitamin E, by HPLC analysis of the hydroxymethyl esters. The end of the induction period is shown by ↓. The rate of chain initiation, $R_i = 2[\text{inhibitor}]/\tau$, measured by Trolox = $10.1 \cdot 10^{-8}$ M s^{-1}. Molar quantities are α-tocopherol, $4.94 \cdot 10^{-8}$ mol; DLPC, $3.20 \cdot 10^{-5}$ mol; ABAP, $2.69 \cdot 10^{-6}$ mol.

vitamin E inhibited, and ABAP-initiated peroxidation of linoleic acid in SDS using oxygen uptake studies [43] but more recently Trolox was reported to have 60–72% of the antioxidant efficiency of vitamin E in this medium using a spectral analysis of diene formation from linoleic acid [44].

Next we compare the effect of vitamin E with Trolox on the c,t/t,t ratios during the inhibition period when peroxidation of DLPC liposomes is initiated in the lipid phase by the lipid-soluble initiator, ADVN. Typical results of these c,t/t,t analyses are given in Fig. 7 for vitamin E-inhibited peroxidation and Fig. 8 for Trolox-inhibited peroxidation. The results for the vitamin E inhibited, ADVN initiated peroxidation are remarkably similar to those during the ABAP initiated reaction (Fig. 5) both in the extrapolated c,t/t,t ratios, 6.2 when using ADVN and 6.6 when using ABAP, and in the slopes of the two plots.

Significant differences result for the ADVN-initiated, Trolox-inhibited experiments compared with those inhib-

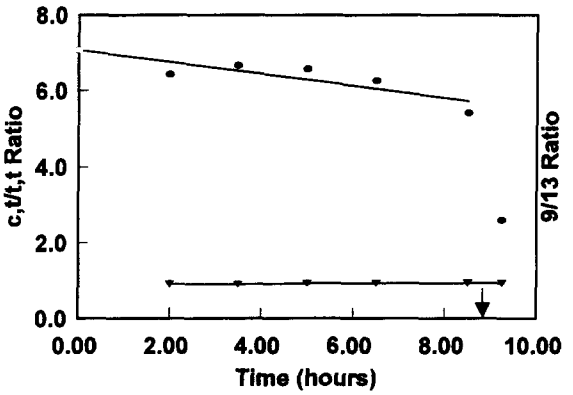


Fig. 6. *Cis,trans* to *trans,trans* (c,t/t,t) linoleate hydroperoxides (●) and 9/13 ratios (▼), from peroxidation of DLPC, initiated by ABAP and inhibited by Trolox. The end of the induction period is shown by ↓. The rate of chain initiation, $R_i = 9.63 \cdot 10^{-8}$ M s^{-1}. Molar quantities are Trolox, $3.56 \cdot 10^{-8}$ mol, DLPC, $3.20 \cdot 10^{-5}$ mol; ABAP, $2.70 \cdot 10^{-6}$ mol.

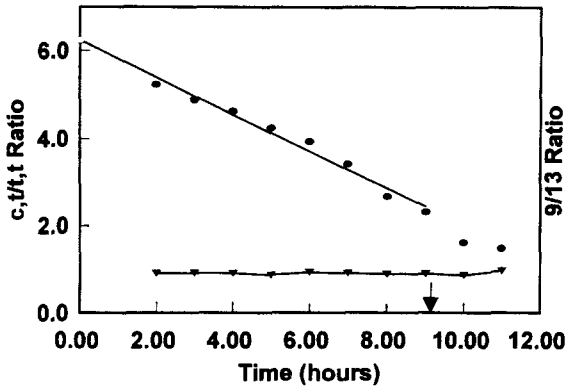


Fig. 7. *Cis,trans* to *trans,trans* (c,t/t,t) linoleate hydroperoxides (●) and 9/13 ratios (▼), from peroxidation of DLPC, initiated by ADVN and inhibited by vitamin E. The end of the induction period is shown by ↓. The rate of chain initiation, measured using Trolox = $7.45 \cdot 10^{-8}$ M s^{-1}. Molar quantities are α-tocopherol, $3.33 \cdot 10^{-8}$ mol; DLPC, $3.10 \cdot 10^{-5}$ mol; ADVN, $3.25 \cdot 10^{-6}$ mol.

ited by vitamin E, initiated by ADVN or ABAP. The extrapolated c,t/t,t ratios exhibit a significant drop in value. Two examples illustrate this result.

In one example, the combination of ADVN initiation and inhibition by Trolox was used for controlled peroxidation of the monomer methyl linoleate solubilized in saturated (non-oxidizing) dimyristoylphosphatidylcholine (DMPC) MLV liposomes. Results, plotted in Fig. 8, show that the extrapolated c,t/t,t ratio drops to 1.3. A second experiment (not shown), employing the combination of ADVN initiation and Trolox inhibition during inhibited peroxidation of DLPC, gave an extrapolated c,t/t,t ratio of 3.5. These results indicate that Trolox is not an efficient antioxidant, when compared to that of vitamin E, when peroxidation is initiated in the lipid phase.

The sites of oxygen attack at the linoleate chain, 9 versus 13, is also of interest because of the polar property of the intermediate peroxyl radicals formed (see Discussion). There does not appear to be a significant preference for the site of oxidation. In the Figs. 5–8, the 9/13 ratios are approximately unity (usually 0.9–1.0) throughout the course of the inhibition period.

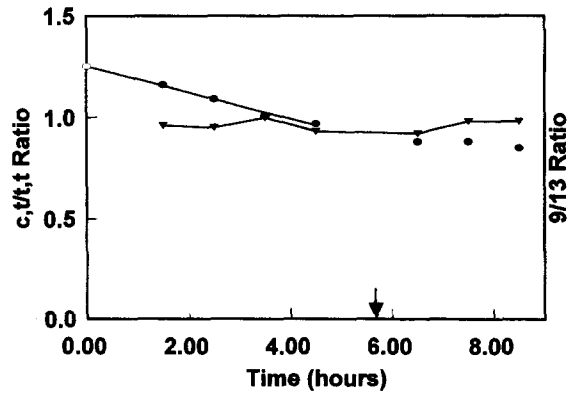


Fig. 8. *Cis,trans* to *trans,trans* (c,t/t,t) linoleate hydroperoxides (●) and 9/13 ratios (▼), from peroxidation of methyl linoleate in DMPC, initiated by ADVN, inhibited by Trolox. The end of the induction period is shown by ↓. The rate of chain initiation = $8.37 \cdot 10^{-8}$ M s^{-1}. Molar quantities are Trolox, $3.43 \cdot 10^{-8}$ mol; methyl linoleate, $6.06 \cdot 10^{-5}$ mol; DMPC, $9.66 \cdot 10^{-5}$ l; ADVN, $3.35 \cdot 10^{-6}$ mol.

4. Discussion

Trolox is a remarkable antioxidant which is known to trap two membrane lipid peroxyl radicals per molecule of Trolox [35] (e.g., its stoichiometric factor is 2) despite the fact that it appears to reside mainly in the aqueous phase. The current sensitive partitioning data using [^{14}C]Trolox are in general agreement with the earlier results on partitioning using other methods (e.g., spectroscopic), where up to 30% partitioning into egg lecithin ULV was found [35]. The fact that Trolox partitions more readily into unilamellar egg lecithin vesicles (at least 40%) than in multilamellar vesicles (25%) indicates that it does not readily distribute itself uniformly into all layers of MLV. This is significant when evaluating its quantitative behaviour as an antioxidant in such heterogeneous systems.

Further insight into the dynamics of the antioxidant action of Trolox is obtained by following the partitioning of total ^{14}C-radioactivity (Trolox and oxidation products) between the aqueous and lipid phase during the controlled peroxidation of DLPC liposomes. Now the membrane bound total ^{14}C rapidly approaches 40%. This result indicates that an equilibrium is readily established between Trolox (aqueous) and Trolox (lipid) on this time scale. It was important to demonstrate that the consumption of total Trolox and the end of the inhibition period do in fact coincide, within experimental error. This is found to be the case as illustrated in the example shown in Fig. 4. This finding supports the preliminary report [33] on the use of Trolox for quantitative studies of autoxidation kinetics in model membranes and demonstrates that it is a very convenient method to measure the rate of free radical initiation during controlled peroxidation of model membranes by azo initiators.

Product studies of the hydroperoxides formed during the inhibition period provide some additional insight into the antioxidant action of Trolox compared to that of lipid-soluble vitamin E. The high c,t/t,t ratio of derived hydroperoxides during initiation by the water-soluble ABAP shows that Trolox is an efficient inhibitor of peroxidation of membrane lipid linoleate (at least as efficient as vitamin E) and that Trolox is not merely trapping initiator peroxyl radicals. On the other hand, the lower relative hydrogen atom donating ability of Trolox when peroxidation is initiated in the lipid phase of MLV (e.g., Fig. 8) may be due to a lack of uniform distribution of the inhibitor Trolox between all layers of the MLV system. The initiator is presumably uniformly distributed by the co-evaporation method. Thus, under this circumstance, some peroxidation would continue uninhibited in the inner

layers and this allows for build-up of the thermodynamic *trans,trans* isomers resulting in lower c,t/t,t ratios.

Peroxyl radicals are known to be highly polarized [45,46] and attempts have been made to account for the kinetic results of autoxidation in bilayers in terms of the dynamics of such polar peroxyls when they are formed initially in the nonpolar lipid layers of membranes [35,47]. It is postulated that such polar peroxyl radicals would diffuse rapidly from the nonpolar lipid phase, where they are initially formed, towards the polar aqueous surface where they would terminate or be trapped (in our experiments) by a water-soluble inhibitor (e.g., Trolox). Such a diffusion-trapping mechanism would account for the very effective action of Trolox when initiation occurs with the water-soluble initiator, ABAP. Such a mechanism might also be reflected in changes in the substitution pattern of the 9- and 13-hydroperoxides formed with the combination of ABAP and Trolox, especially for the Trolox-inhibited peroxidation of methyl linoleate in DMPC. In this case the linoleate is not bonded to the PC and derived peroxyl radicals might diffuse more freely. However, in this as in all experiments, the 9/13 ratios are in the range 0.9 to 1.0. A similar observation was made earlier when α-tocopherol was the inhibitor [42]. Equivalent yields of the 9- and 13-linoleate hydroperoxides under all conditions in our inhibition experiments [1], including those inhibited by Trolox, may be due to two related phenomena. First, if diffusion of peroxyls to the aqueous phase occurs in membranes, it may be faster than the bimolecular hydrogen transfer from inhibitors so that the two peroxyls at positions 9- and 13- are trapped at the same rate by Trolox. Second, the trapping of the two peroxyls initially formed at positions 9- and 13- may be equally facile by Trolox because it is distributed in both phases.

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